STOICHEIOMETRY OF *O*-DEMETHYLASE ACTIVITY IN *PSEUDOMONAS AERUGINOSA*

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1. Introduction

Enzymic cleavage of aromatic ethers was first demonstrated by Axelrod [1] to require an electron donor and molecular oxygen as co-substrates, in liver microsomal preparations. However the stoicheiometric relationships of the reactants in ether cleavage has been difficult to establish due to the extensive oxidation of the electron donors (NADH or NADPH) by the microsomal electron transport chain which terminates with cytochrome *P*-450 [2].

Enzymic preparations have recently been obtained from micro-organisms that demethylate aromatic methyl ethers and these have similar requirements for electron donors and molecular oxygen [3–5]. From their studies on the vanillate *O*-demethylase of *Pseudomonas fluorescens*, Cartwright and Smith [3] suggested that the reaction consumes only one atom of oxygen per mole of vanillate with concomitant formation of protocatechuate and formaldehyde. The data presented here for vanillate *O*-demethylase from *Pseudomonas aeruginosa* do not support the results of Cartwright and Smith but are in accord with the general stoicheiometric relationships of mono-oxygenase (mixed function oxidase) catalyzed reactions:

$$A + O_2 + NADH_2 \longrightarrow AO + NAD + H_2O \qquad (1)$$

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2. Materials and methods

Ps. aeruginosa T1 was grown in mineral salts media [6] supplemented with 0.15 vanillic acid, 0.1 yeast extract, and 0.1 bacto peptone at 30°. Harvested cells were disintegrated after suspension in two volumes of 50 mM tris-HCl buffer, pH 7.6 in a French Press. The extracts were treated with DNase and RNase and centrifuged at 17,000 *g* for 20 min and 100,000 *g* for 2 hr. Oxygen consumption was measured either polarographically with a Clark oxygen electrode or by differential manometry. Simultaneous measurements of oxygen and extinction were made in the cuvette constructed by Ribbons, Smith and Hewitt [7]. Details of individual experiments appear in the legends to the figures.

3. Results and discussion

Concentrated French-press extracts of vanillate-induced *Ps. aeruginosa* T1 [6], which were clarified by centrifugation at 17,000 *g*, rapidly oxidize vanillate to β-ketoadipate in the presence of excess reduced NADH. Measurements of the oxygen consumed during vanillate oxidation were complicated by high levels of respiration shown by both endogenous substrates and added NADH. 100,000 *g* supernatants of these extracts contained little of the NADH oxidase activity and were suitable for determining the stoicheiometry

Table 1
Stoicheiometry of *O*-demethylase with extracts of *Ps. aeruginosa* T1.

Substrate supplied (nmoles)	O_2 consumed (nmoles	moles/ mole substrate)	NADH oxidized (nmoles	moles/ mole substrate)	Phenol formed (nmoles	moles/ mole substrate)	Formaldehyde formed (nmoles	moles/ mole substrate)
Vanillate								
* 175	335	1.91	171	0.98	metabolized		N.D.	
† 5000	9700	1.94	N.D.		metabolized		3000	0.6
m-Methoxybenzoate								
* 250	228	0.91	238	0.95	213	0.95	N.D.	
† 5000	4700	0.94	N.D.		4550	0.91	2250	0.45
Veratrate								
* 250	224	0.9	240	0.96	225	0.95	N.D.	
† 5000	4800	0.96	N.D.		4700	0.94	3000	0.6
Protocatechuate								
* 250	238	0.95	N.D.		–		N.D.	
† 5000	5200	1.04	N.D.		–		N.D.	

* Combined spectrophotometric-polarographic assay.
† Manometric assay.
N.D. not determined.

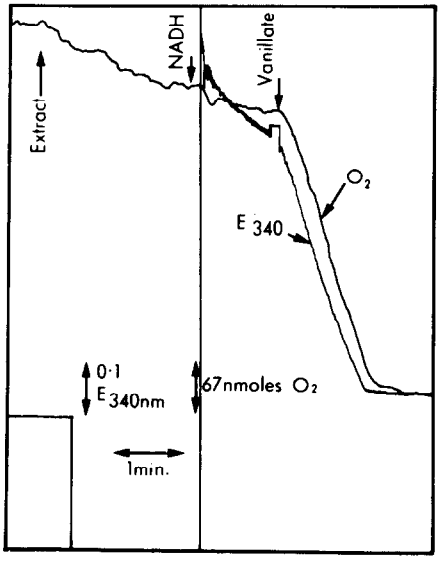


Fig. 1. Simultaneous polarographic and spectrophotometric assay of vanillate oxidation by extracts of *Ps. aeruginosa.* The cuvette contained: 50 mM tris-HCl buffer, pH 7.0 (2.5 ml); additions of extract, G1S (0.2 ml); 25 mM NADH (20 μl); and 25 mM vanillate (7 μl) were made as indicated. Temperature, 30°. Time sequence left to right.

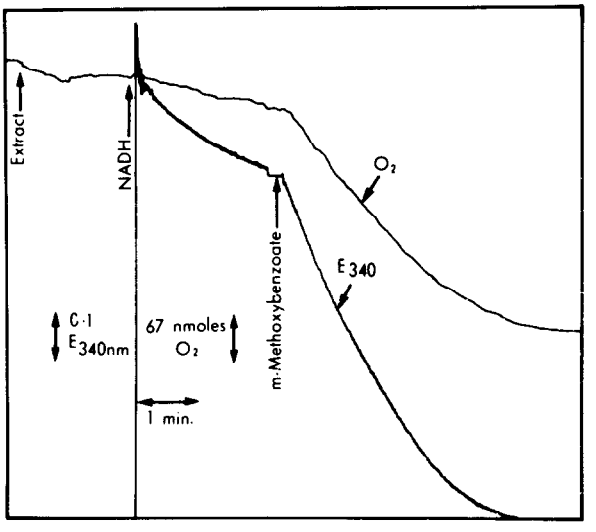


Fig. 2. Simultaneous polarographic and spectrophotometric assay of *O*-demethylase activity by extracts of *Ps. aeruginosa.* The cuvette contained: 50 mM tris-HCl buffer, pH 7.0 (2.5 ml); and additions of extract G1S (0.2 ml); 25 mM NADH (20 μl) and 25 mM veratrate (10 μl) as indicated. Temperature, 30°. Time sequence left to right.

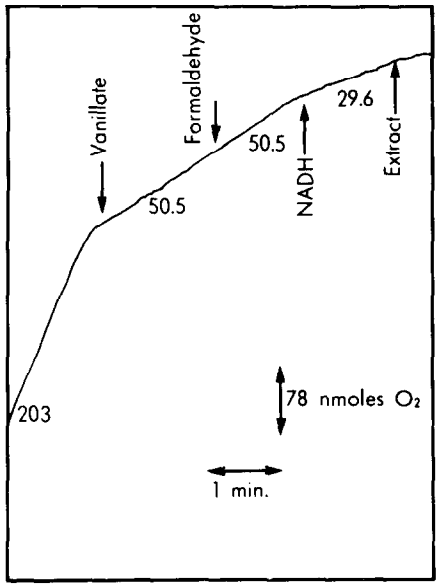


Fig. 3. Formaldehyde and vanillate oxidation assayed polarographically by extracts of *Ps. aeruginosa*. The cuvette contained: 50 mM tris-HCl buffer, pH 7.2 (3 ml); extract A1 (0.1 ml) and sequential additions of 25 mM NADH (40 μl); 25 mM formaldehyde (40 μl) and 25 mM vanillate (10 μl) as indicated. Temperature, 30°. Time sequence right to left. Figures appearing quote O_2 consumption rates (nmoles O^2/min).

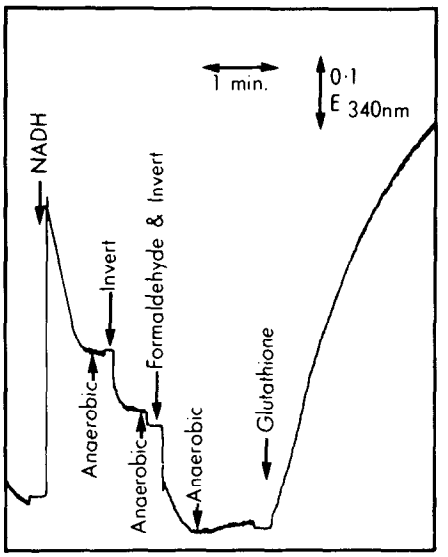


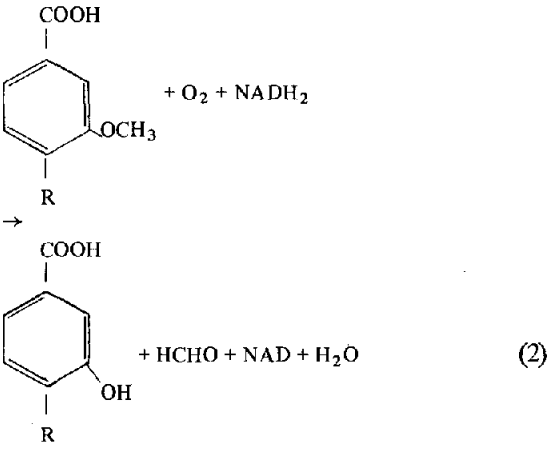
Fig. 4. Formaldehyde dehydrogenase assayed by NAD reduction in extracts of *Ps. aeruginosa*. Each cuvette contained: 50 mM tris-HCl buffer, pH 7.2 (2.5 ml); extract C (0.2 ml) and 25 mM NADH (20 μl) which was oxidized within 2 min. A second addition of 25 mM NADH (10 μl) was made as indicated and only a portion of this was oxidized as the cuvettes had now become anaerobic. This was further demonstrated by inverting the cuvettes and again measuring the decrease in NADH concentration. 25 mM Formaldehyde (100 μl) was added and the absorbance recorded for 1.5 min. 30 mM Glutathione (100 μl) was added as indicated. Temperature, 30°. Time sequence left to right.

of the oxidations. Fig. 1 shows the simultaneous assay of oxygen consumption and NADH oxidation during vanillate oxidation by such preparations. The 3-*O*-demethylation of two substrate analogues *m*-methoxybenzoate and veratrate (3,4-dimethoxybenzoate) is also catalyzed by these extracts (fig. 2). Further metabolism of the hydroxybenzoates formed from these analogous substrates does not occur and *m*-hydroxybenzoate and *iso*-vanillate accumulate quantitatively (table 1). Their identity was confirmed by thin layer chromatography, U.V. and I.R. spectroscopy of the isolated products. The values for O_2 consumption, NADH oxidation and product formation are also shown in table 1. Protocatechuate, the presumed product of vanillate oxidation, is oxidized by protocatechuate 3,4-oxygenase (0.3 μmoles O_2 /min/mg of protein) with the expected oxygen consumption (1 mole O_2/mole of protocatechuate) to β-ketoadipate; and in diluted extracts the intermediate formation of β-carboxy-*cis-cis* muconate was observed by spectral assays.

Formaldehyde and vanillate oxidation rates (measured by oxygen consumption) by the 17,000 *g* supernatants of extracts are compared in fig. 3. This clearly shows that formaldehyde oxidation (linked to oxygen) does not contribute significantly to the observed oxygen stoicheiometry of the demethylase reaction. Formaldehyde dehydrogenase is present in the 10,000 *g* supernatants (assayed by NAD reduction) and is almost entirely dependent upon reduced glutathione as observed by Strittmatter and Ball [8] and Cartwright and Smith [3]. The relatively slow rate of formaldehyde oxidation by unsupplemented 100,000 *g* supernatants is shown in fig. 4 in reaction mixtures that had been made anaerobic by NADH oxidation. It is seen that in the absence of glutathione, formaldehyde oxidation can contribute only negligible values to the stoicheiometric relationships shown in table 1, for both O_2 and NADH consumption. Recovery of formaldehyde from ether oxidation was not quantitative (table 1).

Formate dehydrogenase activity (40 nmoles O_2/min/mg of protein) is located in the pellet sedimented at 100,000 *g* and is not stimulated by NAD.

Thus, the data presented with these unfractionated cell free extracts support the following quantitative relationships for *O*-demethylase activities:



(2)

Vanillate R=OH
m-Methoxybenzoate R=H
Veratrate R=OCH_3

These are typical for mono-oxygenases and differ from those previously reported [3].

In agreement with Cartwright and Smith [3], we find that only concentrated crude extracts have demethylase activity, and NADPH can readily replace NADH as electron donor. Transhydrogenase activity present in these extracts could account for this substitution. However we cannot confirm that activity is lost by dialysis, nor is demethylase activity stimulated by freshly prepared reduced glutathione; reduced glutathione is necessary only for formaldehyde oxidation with these extracts. Extracts of *Pseudomonas testosteroni* also demethylate 3-methoxy-substituted benzoates with identical stoicheiometry shown for *Ps. aeruginosa* but the ether substrate specificity is somewhat different.

Acknowledgement

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